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Review

The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release

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Abstract

HIV accessory genes are expressed throughout the viral life cycle and regulate wide-ranging aspects of virus replication including viral infectivity (Vif and Nef), viral gene expression (Vpr) and progeny virion production (Vpu). While in many cases the molecular basis of accessory protein function is not fully understood, a consensus is emerging that these viral products are generally devoid of enzymatic activity and instead act as multifunctional adapters, subverting normal cellular processes to serve the needs of the virus. This review focuses on presenting our current knowledge of the HIV-1-specific Vpu protein and its essential role in regulating viral particle release, viral load and expression of the CD4 receptor.

Keywords: HIV; Vpu; Accessory genes

1. Introduction

In addition to the prototypical retroviral *gag*, *pol*, and *env* genes, primate lentiviruses, including HIV, encode a number of so-called accessory genes that, contrary to what their name suggests, perform essential functions during the viral life cycle. The term accessory genes was coined following the finding that their inactivation resulted in little or no impairment of virus replication in continuous cell lines [1,2]. However, subsequent studies in vivo or in primary cell types susceptible to HIV infection demonstrated that accessory gene products can dramatically change the course and severity of the viral infection [3].

While the *vif*, *vpr*, and *nef* accessory genes are expressed in most HIV-1, HIV-2 and simian immunodeficiency virus (SIV) isolates, the *vpu* gene is found exclusively in HIV-1 (Fig. 1), with the notable exception of SIVcpz and SIVgsn [4,5]. However, certain isolates of HIV-2 have been shown to compensate for the absence of a bona fide *vpu* gene through functionally equivalent activities in their Env glycoprotein [6–8]. The Rev-dependent bicistronic mRNA that encodes Vpu also contains the downstream Env ORF, which is translated by leaky scanning of the Vpu initiation codon [9]. In primary isolates, the *vpu* gene is not always functional due to the presence of mutated initiation codons or internal deletions [10], suggesting a mechanism by which Vpu expression is regulated by the virus [11].

2. Structure of the Vpu protein

Vpu is an 81-amino acid type 1 integral membrane protein [2,12]. Residues 1–27 constitute the N-terminal hydrophobic membrane anchor, followed by 54 residues that protrude into the cytoplasm (Fig. 2). A highly conserved region spanning residues 47–58 contains a pair of serine residues that are constitutively phosphorylated by casein kinase II [13]. The Vpu cytoplasmic domain contains a high proportion of charged residues, which include a membrane-proximal stretch of basic residues followed by a series of acidic residues in the C-terminal part of the protein that confer an overall negative electrostatic charge to the molecule (Fig. 2). Initial attempts to resolve the structure of the Vpu protein were hampered by the presence of the N-terminal hydrophobic membrane anchor domain, which made the protein highly insoluble in aqueous solutions. Investigators, therefore, focused on partial structures using synthetic peptides

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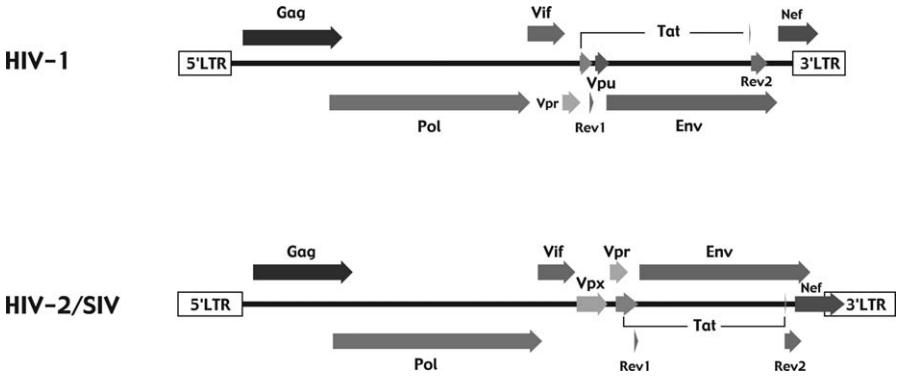

Fig. 1. Map of prototypic HIV-1 and HIV-2/SIV proviruses showing the location of the *vpu* gene.

corresponding to the hydrophilic region (residues 27–81) or fragments thereof. In addition, the inability to successfully crystallize Vpu made it necessary to use circular dichroism and proton NMR spectroscopy in solution to determine the structure of the Vpu cytoplasmic domain. Using synthetic peptides, such techniques detected 2 discrete α-helical structures encompassing amino acid positions 35–50 and 58–70, respectively, separated by a flexible segment containing the two conserved phosphorylated serine residues [14–16]. The Vpu helix 1 is amphipathic with hydrophobic, basic and acidic residues clustered along the axis of the helix (Fig. 3A). The same is true for helix 2, albeit to a less striking degree. Fig. 3B shows a model of the current tertiary fold structure of the Vpu cytoplasmic domain as derived from solution NMR data [17]. Subsequent and still ongoing solid-state NMR studies on oriented phospholipid bilayers aim at providing a better understanding of the structure of Vpu in a more physiological environment. Such techniques were applied to both the transmembrane domain alone and full-length Vpu to yield the current working model of Vpu topology in membranes. Using ^{15}N-labeled peptides encompassing residues 1–27, it was determined that the transmembrane domain forms a stable helical structure with a tilt angle of approximately 15–30° relative to the plane of the membrane [18,19]. These data, along with the extensive structure information available for the Vpu cytoplasmic tail in solution has led to a model of Vpu topology in a membrane environment as depicted in Fig. 3C. The membrane-spanning N-terminal domain forms a stable α-helix connected to the soluble cytoplasmic tail by a short unstructured fragment. A string of positively charged residues within that flexible arm would allow interactions with the negatively charged lipid surface. The hydrophobic side of the helix 1 in the cytoplasmic domain is likely partially buried in the lipid bilayer, exposing the hydrophilic (or charged) side to the cytoplasm. The flexible region joining the cytoplasmic helices 1 and 2 appears to form a loop pointing away from the membrane, mostly due to the acidic nature of the two conserved phosphorylated serine residues [20,21]. The orientation of the second cytoplasmic helix relative to the membrane and the first cytoplasmic helix is still under debate. However, we favor a model where helix 2 extends away from the lipid bilayer for two reasons: first, the constitutively phosphorylated serine residues at positions 52 and 56 do not favor proximity with the plane of the membrane. Second, the strongly negative electrostatic charge of helix 2 makes it unlikely that interactions with the positively charged lipid surface would be favored.

One of the limitations of all of these studies is that they are performed on highly purified Vpu protein. However, biochemical studies have revealed that in HIV-infected cells Vpu interacts with a number of host factors, including CD4 or β-TrCP (see below). It is likely that the interaction of Vpu with such host factors affects its tertiary structure and in particular the orientation of the cytoplasmic helices. Also, homo-oligomerization of Vpu, which was first described by chemical cross-linking experiments [22] but which is not

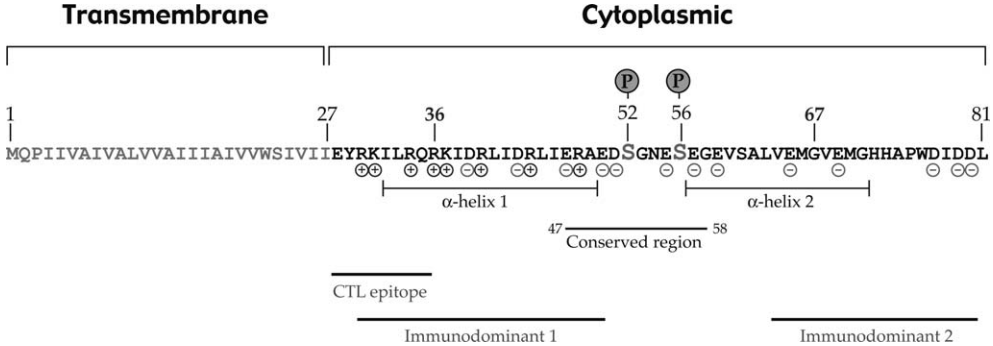

Fig. 2. Annotated sequence of the HIV-1 (NL4-3) Vpu protein. The + and – symbols represent the global charge of the amino acid residues depicted. The two highly conserved and phosphorylated (P) serines residues are indicated at positions 52 and 56. The location of the two alpha-helical structures and the three immunodominant epitopes is also indicated.

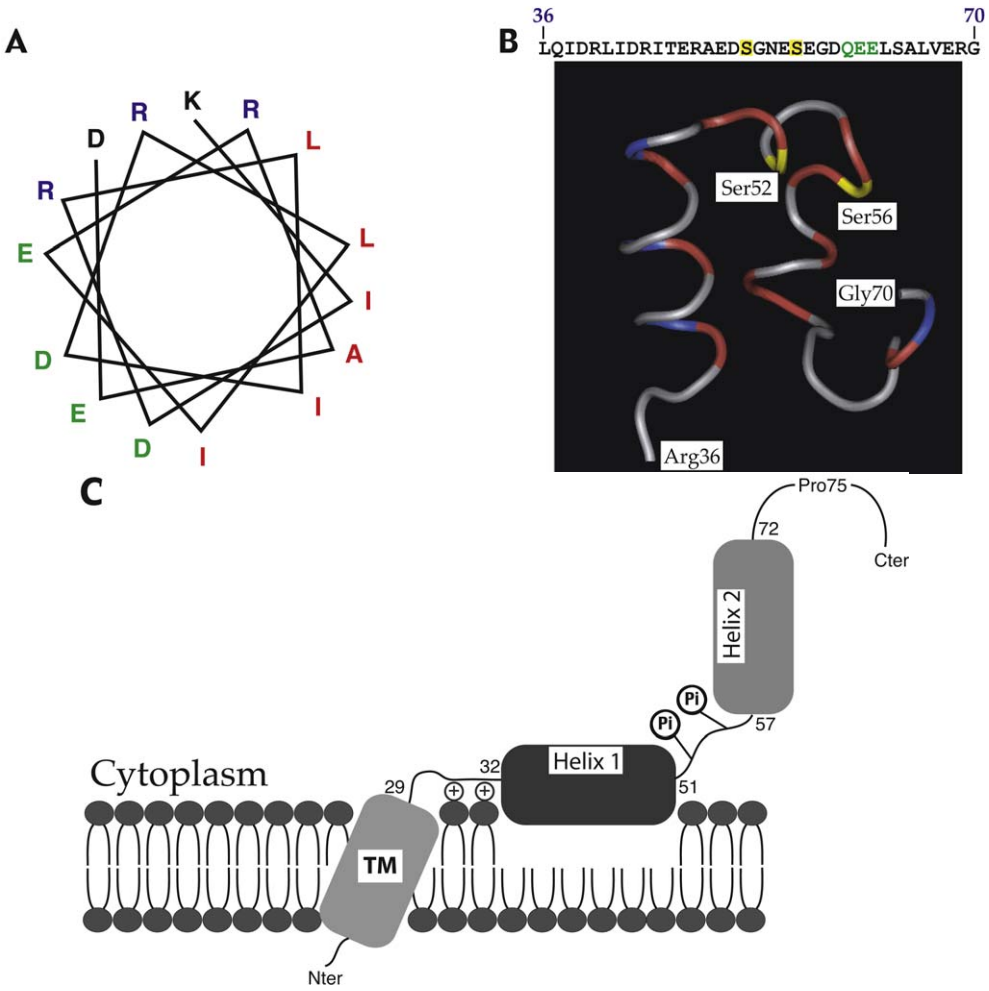

Fig. 3. Vpu structure. (A) Wheel diagram of Vpu residues K_{37}-D_{51} showing the amphipathic nature of helix 1 (adapted from [20]). Negatively charged residues are depicted in green, positively charged residues in blue and hydrophobic residues in red. (B) Tertiary structure of the Vpu cytoplasmic domain residues 36–70 from HIV-1 SF162 (corresponding to residues 36–67 in Fig. 2). A 3-residue insertion not present in the NL4-3 Vpu sequence is shown in green. The Cn3D software was used to generate the 3D structure from the Vpu NMR coordinates (PDB code 5933) published by Willbold et al. [17]. (C) Model representation of the Vpu secondary structure as deduced from available NMR and modeling data.

addressed in current structure models, may significantly impact on the overall conformation of Vpu in membranes.

3. Biological activities of the Vpu protein

3.1. Vpu-mediated degradation of the CD4 receptor

Receptor interference is a hallmark of retroviral infections that involves specific removal of the cellular receptor used for entry into the host. HIV-1 has been shown to effectively interfere with the transport, stability and cell-surface localization of its specific receptor, CD4 [23]. The gp160 envelope glycoprotein precursor (Env) and Vpu both significantly contribute to the viral effort to downregulate CD4. Gp160 is a major player in CD4 downmodulation that can, in most instances, quantitatively block the bulk of newly synthesized CD4 in the endoplasmic reticulum (ER) [23–25]. However, this strategy has two principal shortcomings. First, in contrast to Nef, Env is unable to remove pre-existing CD4 molecules that have already reached the cell surface. Second, the formation of CD4–gp160 complexes in the ER blocks the transport and maturation of not only CD4 but of the Env protein itself [26]. In cases where equimolar amounts of CD4 and Env are synthesized, this could lead to the depletion of cell-surface Env and thus the production of Env-deficient, non-infectious virions [27,28]. An important function of Vpu is to induce the degradation of CD4 molecules trapped in intracellular complexes with Env, thus allowing gp160 to resume transport toward the cell surface [29]. In Vpu-expressing cells, CD4 is rapidly degraded in the ER, and its half-life drops from 6 h to approximately 15 min [30]. The importance of ER localization for CD4 susceptibility to Vpu-mediated degradation suggests that cellular factors essential for CD4 degradation are located in the ER [31]. Indeed, co-immunoprecipitation experiments showed that CD4 and Vpu physically interact in the ER and that this interaction is essential for targeting CD4 to the degradation pathway [32]. Mutagenesis studies delineated a domain extending from residues 416 to 418 (EKKT) in the CD4 cytoplasmic domain

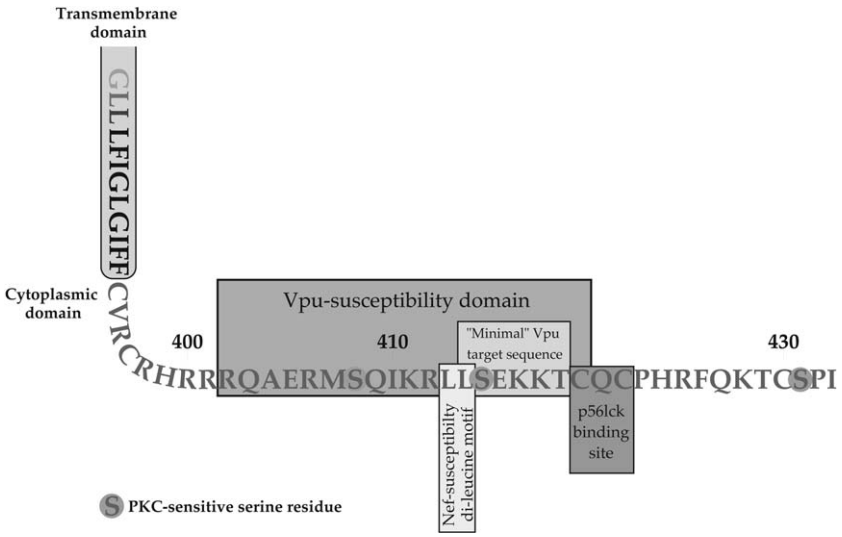

Fig. 4. Functional domains of the CD4 cytoplasmic tail and Vpu target sequence.

required for degradation and Vpu binding [32–35]. Interestingly, the Vpu binding site on CD4 does not include the dileucine motif required for Nef action nor the cysteine residues involved in CD4–Lck interactions (Fig. 4). The domains in Vpu required for CD4 binding are less well defined, suggesting that three-dimensional rather than linear structures are involved. While two conserved serine residues at positions 52 and 56 in the cytoplasmic domain of Vpu are critically important for CD4 degradation [36,37], they are not required for CD4 binding, since phosphorylation-defective mutants of Vpu retained the capacity to interact with CD4 [32]. This finding led to the hypothesis that Vpu binding to CD4 was necessary but not sufficient to induce degradation [32]. The role of the Vpu phosphoserine residues in the induction of CD4 degradation was elucidated when yeast two-hybrid assays as well as co-immunoprecipitation studies revealed an interaction of Vpu with the human beta transducin-repeat containing protein (βTrCP; [38]). Interestingly, Vpu variants mutated at serines 52 and 56 were unable to interact with βTrCP, providing a mechanistic explanation for the requirement for Vpu phosphorylation and strongly suggesting that βTrCP was directly involved in the degradation of CD4 [38].

Structurally, βTrCP shows a modular organization. Similarly to its *Xenopus laevis* homologue [39], human βTrCP contains seven C-terminal WD repeats, a structure known to mediate protein–protein interactions [40]. Accordingly, the WD repeats of h-βTrCP were shown to mediate interactions with Vpu in a phosphoserine-dependent fashion [38]. In addition to the WD repeats, βTrCP contains an F-box domain that functions as a connector between target proteins and the ubiquitin-dependent proteolytic machinery [41]. This domain was of particular significance since there was previous evidence for the involvement of the ubiquitin-proteasome machinery in Vpu-mediated CD4 degradation [42,43]. Although the molecular mechanisms by which Vpu targets CD4 for degradation are now reasonably well defined, it remains unclear how the membrane-anchored CD4 is ultimately brought into contact with cytoplasmic proteasome complexes. A number of proteasome degradation pathways involving βTrCP have recently been deciphered that resemble, at least in part, that of Vpu-mediated CD4 degradation. For example, ubiquitination and proteasome targeting of β-catenin, a member of a transcriptional activation complex involved in development and tumor progression, as well as the NFκB inhibitor IκBα were shown to involve the TrCP-containing SkpI, Cullin, F-box protein (SCF^{TrCP}) E3 complex also involved in CD4 degradation [44–47]. Interestingly, the recognition motif on all known cellular substrates of βTrCP consists of a pair of conserved phosphoserine residues similar to those present in Vpu [38]. These serine residues are arranged in a consensus motif: $DS^pGΨXS^p$; where S^p stands for phosphoserine, Ψ stands for a hydrophobic residue and X stands for any residue. Serine-phosphorylation plays the major regulatory role in the stability of SCF^{TrCP} target proteins. For example, activation of the IκB kinase complex (IKK) by external stimuli such as TNFα induces the serine-phosphorylation of IκBα followed by rapid TrCP-mediated proteasome degradation [48]. Fig. 5 summarizes our current understanding of Vpu-mediated degradation of CD4 and is based on experimental data obtained for both CD4 and IκB degradation. According to our model, phosphorylated Vpu simultaneously binds to CD4 and TrCP and recruits the proteasome degradation machinery through SkpI, an F-box binding protein that associates with TrCP [38]. SkpI, in turn, interacts with Cul-1, a Nedd8-modified entity that provides a docking site for the Cdc34 E2 ubiquitin conjugating enzyme. Cul-1 also interacts with Rbx1, which can bind to both TrCP and Cdc34 and stabilize the E2–E3 complex [49].

While the molecular machinery that assembles around CD4/Vpu complexes is now well defined, it is still not clear how CD4 goes from this targeted state to physical degradation by the cytosolic proteasome. There is only indirect evidence that CD4 ubiquitination precedes its degradation by Vpu [42,43]. It is also not clear at present whether Vpu-induced degradation involves dislocation of CD4 from the

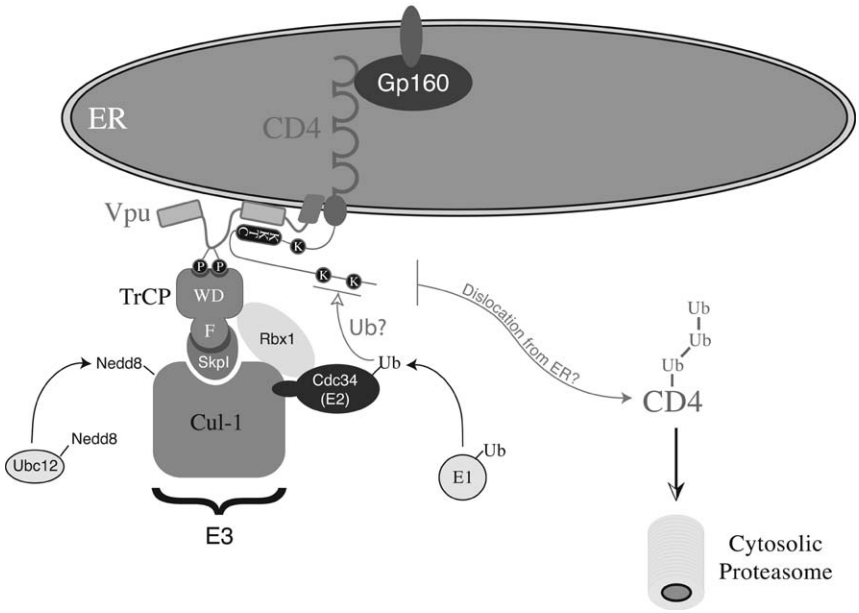

Fig. 5. Model of Vpu-mediated degradation of CD4. The ability of phosphorylated Vpu to interact with both the CD4 cytoplasmic domain and the SCF^{TrCP} E3 complex leads to CD4 degradation by the proteasome. The current model is construed from experimental evidence obtained for both CD4 and IκB degradation by the SCF^{TrCP} [38,92].

ER membrane as shown for other membrane-bound proteasome substrates such as MHC class I heavy chains, whose dislocation from the ER to the cytosol is catalyzed by the human cytomegalovirus US11 gene product [50].

Vpu has one intriguing property that distinguishes it from all other known substrates of βTrCP: its resistance to proteasome degradation. Indeed, while the SCF^{TrCP} usually degrades the serine-phosphorylated protein directly bound to the TrCP WD domains (i.e. Vpu), CD4—bound to the Vpu cytoplasmic domain—is degraded instead. Vpu, therefore, appears to have evolved a decoy mechanism by which Vpu domains that might be targeted for poly-ubiquitination are masked, and those present in CD4 are presented instead to the Cdc34 ubiquitin ligase. This phenomenon has serious implications for the regulation and availability of the SCF^{TrCP} in cells that express Vpu. Indeed, due to the fact that Vpu is constitutively phosphorylated [13], binds βTrCP with high affinity [38] and is not released from the complex by degradation [51], Vpu expression in HIV-infected cells was likely to perturb the physiological function of the SCF^{TrCP} through competitive trapping of TrCP. Examination of the effect of Vpu on the efficiency of SCF^{TrCP}-mediated degradation of IκB and activation of the NF-κB transcriptional activity showed that wild-type Vpu, but not a TrCP-binding deficient serine mutant of Vpu, interfered with TrCP-mediated degradation of IκB [51]. This *trans*-dominant negative effect of Vpu on IκB degradation was shown to lead to inhibition of both HIV- and TNF-α-induced activation of NF-κB [51]. The dysregulation of NF-κB in Vpu-expressing cells has far-reaching consequences, since NF-κB is a central transcription factor that regulates the expression of key cellular genes involved in cell proliferation, cytokine production and the induction of apoptosis [52,53]. Inhibition of NF-κB activity by Vpu might, therefore, contribute to the induction of apoptosis in HIV-1-infected cells [54,55]. This was confirmed experimentally by showing that in a population of Jurkat cells expressing wild-type HIV-1, twice as many cells underwent apoptosis as in cells infected with a Vpu-defective virus [56]. Mechanistically, Vpu was shown to inhibit the NF-κB-dependent expression of anti-apoptotic genes such as Bcl-2 family proteins, leading to enhanced intracellular levels of the apoptosis-promoting caspase-3 [56]. Based on the available experimental evidence, a model for Vpu-induced apoptosis is presented in Fig. 6. In unstimulated cells, NF-κB resides in the cytoplasm in an inactive complex with its inhibitor IκB. Upon stimulation of cells by cytokines such as TNF-α, IκB is rapidly phosphorylated by an IκB-specific kinase, resulting in the rapid degradation of IκB via a TrCP-dependent pathway. In HIV-1-infected cells, Vpu functions as a competitive inhibitor of TrCP. This results in the gradual accumulation of IκB and the progressive impairment of the cell's ability to activate NF-κB. The inhibition of NF-κB blocks the synthesis of anti-apoptotic proteins such as the Bcl-2 family proteins (e.g. Bcl-xl and A1/Bfl-1) or TNF-R complex proteins (e.g. TRAF1). TRAF1 is induced by TNF-α treatment and normally inhibits activation of caspase-8. In Vpu-expressing cells, the levels of TRAF1, in response to TNF stimulation, are reduced and no longer sufficient to inhibit the cytokine-induced activation of caspase-8 [56]. Activated caspase-8 in turn induces the release of cytochrome *c* from the mitochondria. Release of cytochrome *c* is normally inhibited by the Bcl-2 family of proteins. However, in Vpu-expressing cells the levels of Bcl-2 proteins are limiting and no longer sufficient to block cytochrome *c* release [56]. After its release from the mitochondria, cytochrome *c* forms ternary complexes with

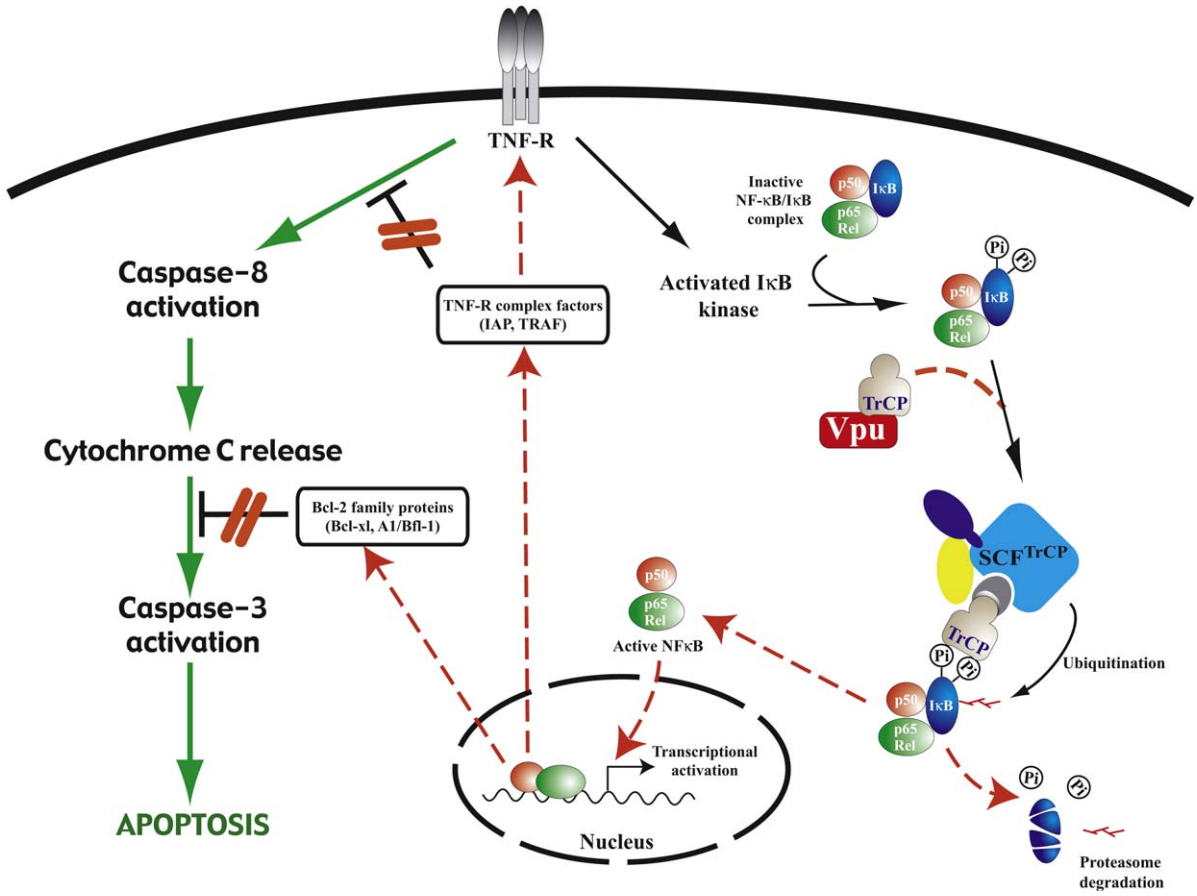

Fig. 6. Model of apoptosis induction by Vpu. Vpu traps the intracellular pool of free TrCP, leading to a lack of activation of NF-κB upon TNF-α stimulation. The steps inhibited by the action of Vpu are indicated in red, and the steps activated by the presence of Vpu are shown in green.

Apaf-1 and caspase-9, resulting in the activation of caspase-3. Active caspase-3 finally triggers a reaction that results in the cleavage of a number of target proteins, including Bcl-2 family proteins, and leads to cell death.

3.2. Vpu-mediated enhancement of viral particle release

In addition to its destabilizing effect on CD4, Vpu mediates the efficient release of viral particles from HIV-1-infected cells [57–59]. These two biological activities of Vpu appear to be mechanistically distinct and involve different structural domains in Vpu. For example, the particle release enhancing activity of Vpu is independent of CD4 and does not require the envelope glycoprotein. Also, mutation of serine residues 52 and 56, which are crucial for CD4 degradation, only partially affect virus release [36,60–62]. In addition, while the determinants for CD4 degradation are all contained in the cytoplasmic domain of Vpu, the transmembrane domain has been shown to play an essential role for the particle release activity [63,64]. It is still debated whether Vpu enhances virus production through a global modification of the cellular environment or through discreet interactions with cellular or viral factors. The finding that Vpu forms ion conductive channels at the cell surface (see below) argues in favor of the former possibility [65]. On the other hand, recent reports suggest that interactions between Vpu and a novel cellular protein (Vpu-binding protein or UBP) may be involved in viral particle production [66]. UBP is a 41-kDa protein that contains four copies of a so-called tetratricopeptide repeat (TPR), a degenerate 34-amino acid sequence involved in protein–protein interactions [67]. Overexpression of UBP was found to abrogate the ability of Vpu to promote viral particle release, suggesting that UBP is a negative factor for virus assembly that needs to be displaced from Gag by Vpu [66]. Examination of the subcellular location of Gag in the presence and absence of Vpu and/or UBP suggests that Vpu may enhance viral particle release by either promoting the transport of viral Gag precursors to the plasma membrane or by increasing the affinity of the N-terminal matrix domain for the plasma membrane lipids [68,69].

3.3. Ion channel activity

Based on the structural similarity of Vpu with the influenza virus M2 ion channel protein, it was speculated that homo-oligomeric complexes of Vpu might possess pore-forming abilities [22]. Indeed, Vpu ion channel activity was experimentally demonstrated in two independent studies by measuring current fluctuations across an artificial lipid bilayer containing either full-length recombinant Vpu protein or synthetic peptides corresponding to the transmembrane

domain of Vpu [65]. In addition, voltage clamp analysis on amphibian oocytes expressing full-length Vpu supports the notion that Vpu forms ion-conductive pores [70]. The Vpu channel appears to be selective for monovalent cations such as sodium and potassium. While some investigators argue that differences in membrane conductance in the presence of Vpu are not due to the opening of an ion channel but rather the result of alterations of the protein membrane composition by Vpu [71], there is an intriguing correlation between the ability of Vpu to form ion conductive channels and its ability to enhance viral particle release in vivo. Indeed, a Vpu mutant bearing a transmembrane domain with a scrambled amino acid sequence lacked ion channel activity and was unable to enhance virus particle release, yet retained full CD4 degradation activity [70]. Nevertheless, how an ion channel activity of Vpu could lead to enhanced viral particle production is still unclear. It is conceivable that the channel activity of Vpu locally modifies the electric potential at the plasma membrane, leading to facilitated formation and release of membrane budding structures. Alternatively, the action of the Vpu channel could induce cellular factors involved in the late stages of virus formation or exclude cellular factors inhibitory to the viral budding process.

While the biochemical and biophysical characterization of Vpu is ongoing, computer simulation has provided a model of how oligomerization of Vpu could lead to the formation of ion conductive membrane pores. In silico modeling suggests that the most stable oligomeric structure of the Vpu transmembrane domain (residues 6–28) is a pentamer with each individual helix tilted approximately 4–6° relative to the membrane plane [72]. Molecular dynamics simulations were then employed to predict the formation, structure and temporal evolution of a pentameric Vpu channel composed of five transmembrane domains. Such computer simulations led to an intriguing model where the Vpu channel could open and close, depending on the positions of the serine residue at position 23 (Ser23) and the tryptophane at position 22 (Trp22) [73]. The channel open configuration would consist of Ser23 facing the lumen of the pore and Trp22 interacting with the lipid polar groups (Fig. 7A). A twisting motion of the Vpu bundle would expose the Trp22 residues to the lumen of the pore and close the channel, due to repulsion of the water molecules by the Trp22 hydrophobic side chains (Fig. 7B). More refined simulations that incorporate full-length Vpu and simulate a voltage across the membrane will be needed to present a full prediction of the structure, behavior and selectivity of the putative Vpu ion channel.

4. The evolution of Vpu biological activities

Although the *vpu* gene is unique to HIV-1, the activity Vpu provides for enhanced viral particle release is not. Indeed, the envelope proteins of several HIV-2 isolates, including ROD10 and ST2, were shown to promote viral particle release in a manner indistinguishable from that of HIV-1 Vpu [6,7]. Both Vpu and the ROD10 Env are functionally interchangeable, and each augments the release of HIV-1, HIV-2 and SIV particles, suggesting a common mechanism of action for these two proteins [8,74]. Due to its innate tendency to form homo-oligomeric complexes, it seems possible that HIV-2 Env, in analogy to Vpu, mediates the release of viral particles through the formation of a membrane pore. This is supported by the fact that Vpu and the HIV-2 Env both require the presence of a functional transmembrane domain for their activity [7,63] and adopt an oligomeric structure favorable to the formation of a membrane pore [22]. Mutagenesis studies have delineated the regions in the HIV-2 Env important for its particle release activity. One study proposed that the C-terminal part of the Env cytoplasmic domain is required for efficient particle release [6]. However, such correlation between the length of the cytoplasmic tail and the presence of particle release-promoting activity could not be confirmed for the ROD10 isolate [75]. In addition, ROD14, a molecular clone of HIV-2 closely related to

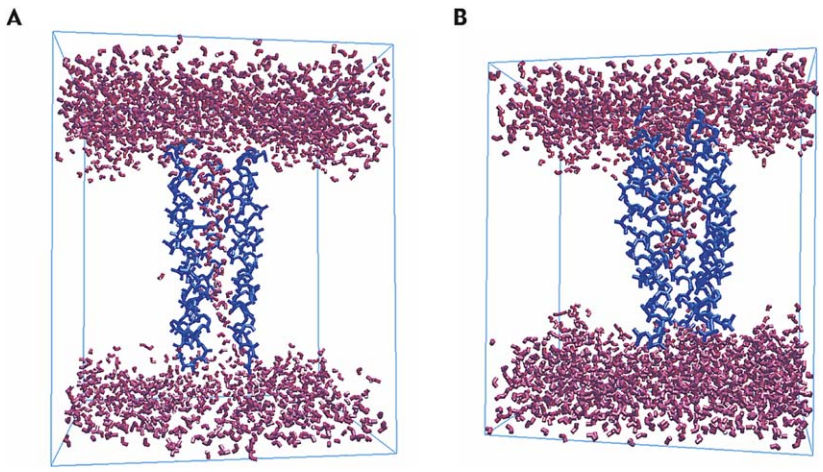

Fig. 7. Molecular dynamics simulation of the Vpu channel. Simulations were performed as described [93]. The figure depicts water molecules (purple) flowing through a pentameric Vpu channel (blue) over time. Panel A shows the state of the channel at the beginning of the run and panel B shows the channel at the end of the run (3 ns). The N-terminus of Vpu is at the top. Adapted from [93] with permission.

ROD10 that originated from the same patient [76], does not support viral particle release irrespective of the length of its cytoplasmic domain [75]. Instead, site-directed mutagenesis revealed that the ability of the HIV-2 ROD Env protein to enhance viral particle release is regulated by a single amino acid substitution (position 598) in the ectodomain of the gp36 TM subunit [77]. Substituting the threonine at that position in the inactive ROD14 Env by the alanine found at the same position in the active ROD10 Env restored full particle release activity to the ROD14 Env in transfected HeLa cells [77].

Unlike Vpu, the HIV-2 Env protein is unable to induce CD4 degradation [8]. The absence of a degradative activity in the ROD10 Env suggests that this additional function may have evolved in Vpu from the ancestral particle release activity in response to increased affinity between the HIV-1 Env and CD4 [8,29]. Additional evidence in favor of this hypothesis comes from examining the sequence of SIVcpz isolates. The serine residues at positions 52 and 56 essential for interaction with TrCP are less conserved in SIVcpz than in the prototypical subtype C HIV-1 isolates [78]. The Vpu proteins from SIVcpz isolates are, therefore, unlikely to induce CD4 degradation. Since SIV isolates bearing a pseudo *vpu* gene are viewed as potential ancestors of HIV-1, it is tempting to speculate that the CD4 degradation ability of Vpu appeared late in the evolution of HIV-1.

5. Vpu contributes to HIV-1 pathogenesis by raising viral loads

Vpu is one of the least antigenic proteins of HIV-1. Despite the delineation of two immunodominant B–cell epitopes in Vpu (Fig. 2), only 20–30% of patients tested exhibit detectable immune response to Vpu [79,80]. Vpu also appears to be a poor target for cytotoxic T cells. Although a major CTL epitope was identified between residues 28 and 36, less than 3% of patients screened have detectable Vpu-specific CTL responses against this peptide [81]. There are conflicting reports on the possible link between the presence of Vpu-specific antibodies in patients and disease progression. One early study found no temporal relationship between the presence or absence of Vpu antibodies and the onset of HIV-1-related disease [82]. In contrast, Kusk et al. [79] found a statistically relevant correlation between the presence of antibodies against the immunodominant epitope 31–50 and a late disease stage characterized by CD4+ T-cell counts of <400 cells/μl. However, the notion that antibodies against Vpu are a valid marker of disease progression is further challenged by the finding that in a cohort of 243 HIV-1-infected patients, Vpu-specific antibodies against another immunodominant epitope (residues 64–81) were actually more prevalent in individuals in the early stages of disease [80]. A possible explanation for these divergent sets of data may be that the Vpu sequence appears to be the most variable among all HIV-1 genes [83]. Indeed, experimental methods employed to detect both humoral and CTL activities rely on reactions against synthetic peptides whose sequences are based on the consensus of cloned viruses. Given the rate of variability of the immunodominant epitopes in Vpu, it is entirely possible that such diagnostic assays give false-negative readouts when used against widely divergent Vpu sequences. While it may be possible to better address the question of the immune response against Vpu by adapting the detection peptides to geographical clusters of Vpu subtypes, available evidence indicates that the presence of antibodies against Vpu is not a reliable predictor of disease stage or outcome.

Stronger lines of evidence point to a role of Vpu in HIV pathogenesis. Studies in pig-tailed macaque using SIV/HIV chimeric viruses (SHIV) have shown that mutation of the *vpu* initiation codon rapidly reverts to give rise to a functional *vpu* ORF [84]. Such reversion occurs as early as 16 weeks post infection and correlates with a phase of profound loss of CD4-positive cells [85]. Similar results were obtained in cynomolgus monkeys, where the presence of Vpu was correlated with a vast increase in the plasma viral RNA levels 2 weeks post-infection [86]. The increased viral fitness and pathogenicity conferred by Vpu is bimodal. First, Vpu increases viral loads in the plasma, thereby contributing to viral spread. Second, the higher frequency of de novo infections that results from these higher viral loads leads to increased rates of mutations in the *env* gene [86,87]. This in turn leads to more rapid and efficient escape from neutralizing antibodies and accelerated disease progression [86]. In animals infected with viruses where vpu deletions were large enough to prevent reversions, investigators observed long-term non-progressing infections characterized by a lack of circulating CD4+ T cell loss [88]. Finally, studies in pig-tailed macaques showed that in the presence of large deletions in *vpu*, additional mutations in the *env* gene were acquired that partially compensated for the lack of Vpu [89,90]. Although the mechanism by which Env would recapitulate the activity of Vpu in these animals is unclear, it is tempting to speculate that Env might have acquired a particle release activity similar to that displayed by some HIV-1 macrophage tropic isolates [11] and some HIV-2 isolates [6,7].

6. Conclusion: is increasing viral particle release the main goal of Vpu?

As details of Vpu's action on CD4 degradation and particle release emerge, the question arises as to why such apparently unrelated activities have evolved within a single protein. One possibility is that enhancement of particle release and CD4 degradation are two unrelated activities, each performing its own function in the viral life cycle. The main role of the CD4 degradation activity would thus be to liberate envelope protein precursors trapped in intracellular complexes with CD4 [26,29]. As the rate of viral particle production augments, the action of Vpu would guarantee that enough mature envelope proteins are available for incorporation into virions. In addition, it is possible that CD4 degrada-

tion was selected as a supporting feature to the main particle release activity. Indeed, there is experimental evidence that the presence of CD4 at the cell surface actively interferes with the ability of Vpu to promote viral particle release [91]. Another purpose of intracellular degradation of CD4 by Vpu would, therefore, be to prevent interference by cell surface CD4 with the Vpu particle release activity [91].

While Vpu might still be referred to as an accessory protein, there is clear evidence that its role in enhancing viral particle production, downregulating cell-surface CD4 and raising viral loads in vivo is key to the fitness and pathogenesis of HIV-1. It may be too early to call Vpu a viral pathogenesis factor but it is interesting to note that closely related retroviruses such as HIV-2 and SIV with less severe pathogenesis and disease outcome all lack expression of the Vpu protein.

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